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In vitro and *In vivo* Antibacterial Activity of *Spirulina platensis*

Thura M.H. Alyasiri[1,]*, Ithar Kamil Al-Mayaly[2] and Salah, M.M. AL-Chalabi[3]

[1,2] *Department of biology, college of science, Baghdad university, Iraq.*
[3] *Biotechnology Research Center, AL-Nahrain University, Iraq.*

* Corresponding author: Thura M.H. Alyasiri; e-mail: biologistthura@gmail.com

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ABSTRACT

Algae as a natural therapy recently drew attention because its limited side-effects. In the present investigation, after phytochemical analysis of *Spirulina platensis*; different concentrations of *S. platensis* aqueous extracts were investigated *in vitro* and *in vivo* for antimicrobial effect against pathogenic *Pseudomonas aeruginosa* as gram negative bacteria; and *Staphylococcus aureus* as gram positive bacteria, which commonly associated with wound infections. After using Agar well diffusion method, variable inhibition zones were observed at 100mg/ml of extract compared with reference antibiotics (Ceftazidime for *P. aeruginosa* and Azithromycin for *S. aureus*). By using open wounds procedure as a clinically *in vivo* wound model that contaminated with *P. aeruginosa* and *S. aureus*, treatment with the extract showed accelerated epithelial regeneration compared with reference antibiotics, and infected wounds without treatment. Through the results of *in vitro* and *in vivo* experiments, we observed that *Spirulina platensis* aqueous extract has an effective antibacterial activity which a proved by accelerating the wound healing process.

Keywords: Antibacterial; *In vitro*; *In vivo*; *Spirulina platensis*.

1. INTRODUCTION

In recent years, human pathogens have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Undesirable side effects of certain antibiotics and the emergence of previously uncommon infections led the scientists to look for new antimicrobial substance from various sources, especially derived from natural compounds [1].

To date, many chemically unique compounds of fresh water origin with various biological activities have been isolated, and some of them are under investigation while some are being used to develop new pharmaceuticals. There are numerous research on compounds derived from algae with a broad range of biological activities, such as antibiotics [2]. Literature has confirmed that crude and purified molecules derived from algae have been found to display a great amount of antimicrobial effect *in vivo* and *in vivo* against both gram positive and gram negative bacterial pathogens [3]. Cyanobacteria are emerging as an exciting resource for the discovery of new classes of therapeutics [4]. Instead of extracting the vaccine, people could eat the algae directly and let their bodies metabolize the vaccine [5].

Initially *Spirulina* alga was classified as plant kingdom because of plant pigments as well as photosynthesis ability, later it was placed in the bacteria kingdom based on new understanding of its genetics, physiology and biochemical properties [6]. *Spirulina's* cell walls consist of soft mucopolysaccharides, making it easy for the body to digest [7]. Some species of *Spirulina* have been classified as a new record in Iraq, such as *Arthrospria jenner* according to 2014 checklist of algae in Iraq [8]. The present investigation evaluates the

antimicrobial activity of *Spirulina platensis* against pathogenic gram positive and gram negative bacteria.

2. MATERIALS AND METHODS

2.1Aqueous extraction method

Twenty grams of freeze-dried biomass of *Spirulina platensis* was suspended in 200 ml of distilled water, then shaken continuously for 24 hours at 30 ˚C. The mixture was then centrifuged at 5000 rpm for 10 minutes and filtered by Whatman filter paper No.1 to remove the cell debris. The extract was evaporated by rotary evaporator at 35˚C and 60 rpm, and stored at 4°C before use for the experiments [9].

2.2 Phytochemical Analysis detection :

Flavonoids detection solution was prepared by using ethanol and potassium hydroxide [10]. While **Saponins** detection was done according to the method described by [11] by shaken vigorously until the formation of foam. **Alkaloids** procedure was done as described by [12] using Mayer's reagent. **Tannins** detection was carried out by adding 1 % lead acetate to the aqueous extract [13]. **Terpenes** procedure of [14] was used by mixing 1 ml of extract with 2 ml of chloroform and 1 drop of glacial acetic acid; one drop of H_2SO_4 was added and the appearance of brown color was an indicator for the presence of terpenes.

2.3 Determination of antimicrobial activity

2.3.1 *In vitro* antibacterial test

Different concentrations of aqueous extracts (25, 50, 75, 100 mg/ml) were tested by the Agar well diffusion method [15] against two strains of human pathogenic bacteria used in this study *Pseudomonas aeruginosa* as gram negative bacteria; and *Staphylococcus aureus* as gram positive bacteria which were obtained from the biology department/microbiology, Faculty of Science at University of Baghdad.

Preparation of 24 hours activated culture, according to [16] by transferring a full loop of culture from nutrient agar slants to 10 ml of Nutrient broth and incubated at 37 C° for 24 hours. Not that one ml of *S. aureus* suspension containing (5.5 x10^5 cell/ml); while one ml of *P. aeruginosa* suspension containing (7.5 x10^5 cell/ml), Been counted through a total plate count procedure according to [17]. Muller Hinton agar had been inoculated with 0.1 ml of each species of activated microbial suspension, spread with a sterilized spreader to form a lawn of culture, and after 15 minutes holes were made in the seeded agar using a cork borer in diameter size 6 mm [15]. An aliquot of 20 µl from each algae crude extract (25, 50, 75, 100 mg/ml) was added into each well on the seeded medium and allowed to stand on the bench for 1 h for proper diffusion and thereafter incubated at 37C° for 24-48 h. The results inhibition zones surrounding the wells were measured in millimeters (mm) by special ruler. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used for antibiotics activity assay and *in vivo* assays in rats.

2.3.2 Antibiotics activity assay

Antibacterial activities of 100 mg/ml conc. of *S. platensis* aqueous extracts was compared with the positive control (selective antibiotics), including Ceftazidime discs 10µ for *Pseudomonas aeruginosa* and Azithromycin discs 15µ for *Staphylococcus aureus*, with antibiotic potency 10 and 15 µg sequentially (Bioanalyze company)**;** the results were measured after incubated at 37 °C for 24-48 hours [18].

2.3.3 *In vivo* antibacterial test (Open wounds)

Thirty adult rats of both genders weighting (135±5 g) were divided into 6 groups (5 rats/group), were anesthetized with anesthetic ether and shaved at the predetermined site before wounding. Three centimeter long incision wounds were made using a sterile scalpel on the flank area of the animals [19]. The animals were then placed in separate cages to avoid any disturbance. All incisions were infected with 1 ml of bacterial suspension (*S.aureus* or *P.aeruginosa*) and after 24 hours of infection, rats were treated as follows:

Group 1: Infected with *S.aureus;* treated with aqueous extracts of *S. platensis* 100 mg/ml for 7 days.
Group 2: Infected with *S.aureus;* treated with Azithromycin antibiotic (concentration 2% per 15g w/w) for 7 days (as a positive control).
Group 3: Infected with *S.aureus* and left without treatment (as a negative control).
Group 4: Infected with *P.aeruginosa*; treated with aqueous extracts of *Spirulina platensis* 100 mg/ml for 7 days.
Group 5: Infected with *P.aeruginosa;* treated with Ceftazidime antibiotic (concentration 100 mg/ml) for 7 days (as a positive control).
Group 6: Infected with *P.aeruginosa* and left without treatment (as a negative control).

The wound healing activity of the aqueous extracts of *S. platensis* (concentration 100 mg/ml) was measured after 7 days in all groups, with a comparison on the progressing of wound healing which treated with reference antibiotic and infected-non-treated wounds

3. RESULTS AND DISCUSSION

3.1 Phytochemical analysis

Many of the health benefits of *S. platensis* have been linked to its contents, water has been used to achieve a high recovery of these compounds. In this study, phytochemical analysis showed that *S. platensis* aqueous extract contained some active compounds such as Flavonioed, Alkaloied, Terpenes, Glycosides and Saponins except Tannins. Results which were in agreement with those discussed by Kannan et al. [20]. These antioxidants have been reported to play a significant role in the wound healing process by protecting tissues from oxidative damage [21]. Several studies have focused on physiological qualities of some valuable antiviral or antioxidant compounds in blue green algae *Spirulina* [22]. The flavonoids reporting that they are active against several strains like *Staphylococcus aureus* [23]. It was also reported that

Staphylococcus is sensitive to polysaccharides [24] and *Spirulina* showed the highest content of Glycosides which condensation products of sugars. Saponins produce foam upon shaking. Alkaloids comprising of nitrogen bases synthesized from amino acid building blocks with various radicals [25]. Many terpenes are known to be active against a wide variety of microorganisms, including gram-positive and gram-negative bacteria [26]. That may be returned to saponins, which are natural detergents, saponins can prevent adhesion of bacteria to eukaryotic cells *in vivo*, in fact adhesion and colonization are required for the subsequent development of diarrheal disease [27].

3.2 *In vitro* antibacterial results

The results showed susceptibility of both strains (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) for *S. platensis* extract with different concentrations.

Table 1 explains greater values of inhibition zones for both *S. aureus* and *P. aeruginosa* were obtained at the 100 mg/ml of extract.

Table 1: Inhibition zones of bacterial growth at different concentrations of *S. platensis* aqueous extract

Bacterial strains	Inhibition zone diameters (mm) of *S. platensis* aqueous extract Mean ± SE			
	100 mg/ml	75 mg/ml	50 mg/ml	25 mg/ml
Pseudomonas aeruginosa	16±0.12	R	14±0.11	15±0.11
Staphylococcus aureus	19±0.21	16±0.23	14±0.12	12±0.14

Therefore, the concentration 100 mg/ml of extract was taken to compare with positive control (selective antibiotics). The results showed that the efficacy of the extract to inhibit *P. aeruginosa* was at an acceptable degree when compared with the Ceftazidime; 18 mm and 22 mm respectively. As for the *Staphylococcus aureus;* table 2 and fig. 2; it was found that the inhibition zone diameter of the extract was 19 mm, compared with the inhibition zone diameter of Azithromycin antibiotic which was 24 mm. These concluded results may relate to the cell wall of gram-positive bacteria which is less chemically complex than that of the gram-negative-bacteria [28]. And Gram-negative bacteria are resistant due to the barrier of lipopolysaccharides and phospholipids, into which nonspecific pores and specific uptake channels are embedded of their outer membrane [29].

Table 2: Inhibition zones of bacterial growth at 100 mg/ml concentrations of *S. platensis* aqueous extract compared with the antibiotic chosen for each bacteria

Bacterial strains and standard antibiotics		Inhibition zone diameters (mm) Mean ± SE
Pseudomonas aeruginosa	*Spirulina* aqueous extract	18± 0.21
	Ceftazidime antibiotic	22± 0.09
Staphylococcus aureus	*Spirulina* aqueous extract	19± 0.11
	Azithromycin antibiotic	24± 0.04

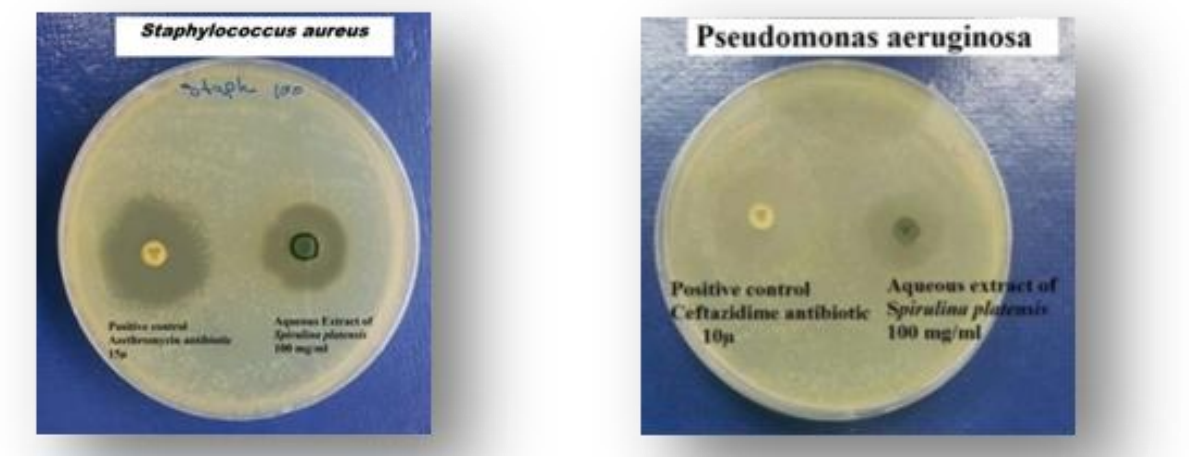

Figure 2: A. Effect of Azithromycin antibiotic and 100mg/ml extract against *S. aureus*; B. Effect of Ceftazidime antibiotic and 100mg/ml extract on *P. aeruginosa*

3.3 *In vivo* antibacterial results (Open wounds)

The goal of the animal models is to investigate the antibacterial activity of the investigational drug [30]. Wound healing is a complex and dynamic process by which cellular structures and tissue layers in a damaged tissue restores itself as closely as possible to its original state [31].

After application of aqueous extracts of *S. platensis* onto infected wound, the results of thirty adult rats showed the inflammation signs, swelling, redness, hotness and some wounds showed a purulent discharge after infected with *S. aureus* and *P. aeruginosa*. But after 7 days of treatment with *Spirulina* aqueous extract 100 mg/ml concentration or with standard antibiotics, there was a complete or accelerated healing, and sometimes observed without or remnant scar tissue, such as fig. 3-a, b. The absence of irritation and pain at the wound site during treatment and the significant increase in the rate of wound shrinkage and wound re-epithelialization is a reflection of good antibacterial potentials of the extract as contained in the *in v*itro and *in vivo* antibacterial assay result*s.* Inhibition of bacteria in the site of the wounds is probably what led to the division and regeneration of cells resulting in acceleration of the healing process.

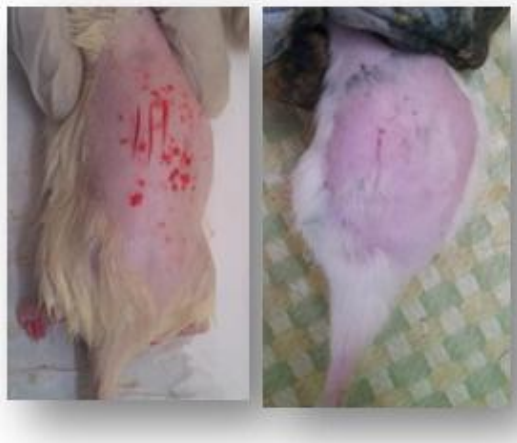

Figure 3-a: Skin of rat infected with *S. aureus* suspension ; ana after 7 days of treated with 100 mg/ml of *S. platensis* aqueous extracts

Figure 3-b: Skin of rat infected with *S. aureus* suspension; and after 7 days of treated with Azethromycin antibiotic (as positive control).

While the third group (negative control) which their wounds were contaminated with ***S. aureus*** bacteria and left without any treatment, fig. 3-c, showed partial healing through the wound area undergoing shrinkage, but formation scar tissue after 7 days, this came compatible with Mohan [32] about Inflammation and healing.

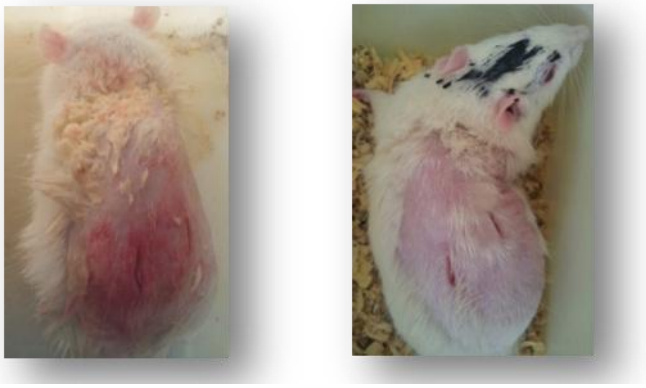

Figure 3-c: Skin of rat infected with *S. aureus* suspension; and after 7 days of left without treatment (as negative control).

Secondly, concerning wounds infected with *P. aeruginosa*, the signs of inflammation also observed locally at the site of the wound; it was more obvious than the wounds of rats contaminated with *Staphylococcus* bacteria. Recovery of tissues was observed in wounds treated with *S. platensis* extract; as shown in fig. 4-a, which were very satisfactory and inspirational result compared with the wounds treated

with Ceftazidime antibiotic as in fig. 4-b in which wound healing was very observed. In the present study, application of *S. platensis* extract accelerated wound healing, wound shrinkage is made possible either due to an enhanced activity of fibroblast in regenerated wound tissue [33]. The wound contraction and healing effects of *S. platensis* extract might be attributed to Stimulate epithelial cell proliferations and angiogenesis are vital for the wound healing process to take place [34]. Wound epithelialization is a process whereby there is epithelial regeneration post wounding with the epithelial cells proliferating and migrating over the wound bed, thereby providing a protective cover for the freshly formed tissues [35]. So *Spirulina* extract might have accelerated epithelial regeneration due to its ability to promote angiogenesis and collagen formation and depositions [36]. The extract was able to recovery wound comparable to the reference drug, Ceftazidime.

These signs were gradually subsided after 7 days; except; the group left without antibiotic treatment or *Spirulina* aqueous extract treatment, as given in fig. 4-c, showed other signs include delays in the proliferative and wound healing process due to the release of free radical and lytic enzymes on the wound site [37]. The delay in the wound healing by these free radicals is achieved by the ability of the free radical to destroy the lipids, proteins and extracellular matrix [38].

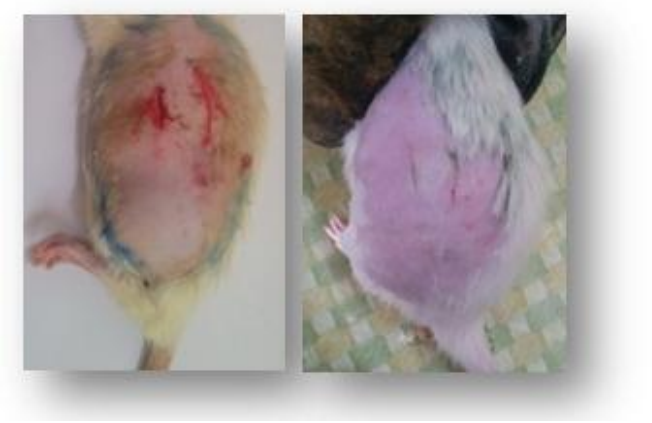

Figure 4-a: Skin of rat infected with P. aeruginosa *suspension; and after* 7 days of treated with with 100 mg/ml of S. platensis aqueous extracts

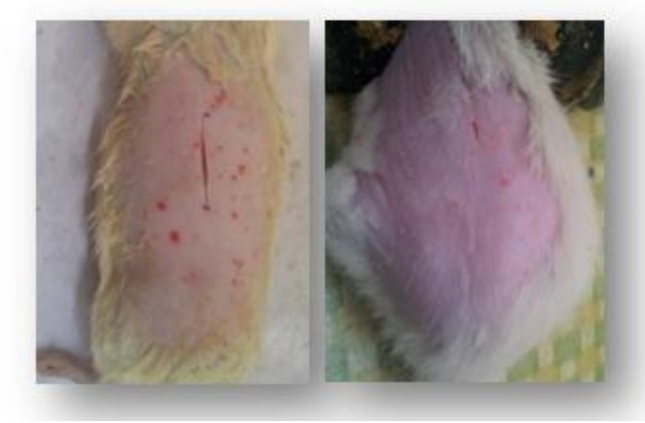

Figure 4-b: Skin of rat infected with *P. aeruginosa* suspension; and after 7 days of treated with with Ceftazidime antibiotic (as positive control)

Figure 4-c: Skin of rat infected with *P. aeruginosa* suspension; and after 7 days of left without treatment (as negative control).

4. CONCLUSION

Iraqi *Spirulina platensis* could be used for management of gram+ve and gram-ve microbial infections because of its antibacterial and antioxidant properties which enhance wound healing by accelerating wound shrinkage and re-epithelialization.

Acknowledgments

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